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HOMOLOGOUS RECOMBINANT GROWTH HORMONE AND CALCIUM METABOLISM IN THE TILAPIA, *OREOCHROMIS MOSSAMBICUS*, ADAPTED TO FRESH WATER

G. FLIK, W. ATSMA, J. C. FENWICK[1], F. RENTIER-DELRUE[2], J. SMAL[3] AND S. E. WENDELAAR BONGA

Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, NL-6525 ED, Nijmegen, The Netherlands, [1]*Department of Biology, University of Ottawa, Ottawa, Ontario, Canada K1N 6N5,* [2]*Laboratoire central de Génie génétique, Université de Liège, Institut de Chimie B6, B-4000 Sart Tilman, Belgium* and [3]*Eurogentec SA, 14 Rue Bois St Jean, B-4102 Seraing, Belgium*

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Summary

Homologous recombinant tilapia growth hormone (rtGH) was tested for its effects on calcium metabolism in freshwater tilapia *Oreochromis mossambicus*. Fish were fed an optimal ration of 5% of their body mass per day. A positive correlation was found between the amount of food given and the branchial calcium influx. In male tilapia, the mean calcium influxes were 5.80 and 11.71 $\mu mol\,h^{-1}\,100\,g^{-1}$ when they were fed 2% and 5% food, respectively. In female fish fed 5% food, the calcium influx was 6.20 $\mu mol\,h^{-1}\,100\,g^{-1}$. Calcium influx *via* the gills was not affected by rtGH. However, in rtGH-treated fish, the net efflux of calcium was lower than in the controls. Apparently, the calcium taken up from the water was more efficiently stored in the body. GH increased the hepatosomatic index and had mild growth-promoting effects (mass and length increases); it increased the total body calcium pool without affecting bone or scale calcium density. The chloride cell density in the opercular epithelium almost doubled after GH treatment. GH did not influence plasma ion composition. Plasma cortisol levels were lower in rtGH-treated fish. A comparison of the roles of GH and prolactin (the products of the prolactin gene family) in calcium regulation of the tilapia led us to conclude that GH has specific calcitropic effects on freshwater tilapia that differ from those of prolactin.

Introduction

A characteristic of the members of the prolactin gene family is their pleiotropic nature. In fish, growth hormone (GH) acts as a growth-promoting hormone, but it also appears to be involved in osmoregulation, stress adaptation and reproduction (Le Bail *et al.* 1991). Growth, osmoregulation, stress adaptation and reproduction are strongly interdependent in fish, and all these processes depend on a well-regulated calcium balance; Ca^{2+} uptake

Key words: tilapia, *Oreochromis mossambicus*, recombinant growth hormone, fresh water, chloride cells, growth, calcium flux.

via the gills is pivotal in calcium handling by fish (Flik *et al.* 1985). The interdependence of these processes and their dependence on GH may underlie the pleiotropic character of the effects of GH in fish.

Studies on the effects of GH in fish have focused on salmonids, eels, cyprinids and tilapia. The emphasis of the work carried out so far has been on the establishment of sensitive assays for the determination of (plasma) levels of the hormone (e.g. Fryer, 1979; Cook *et al.* 1983; Bolton *et al.* 1987; Le Bail *et al.* 1991; Ayson *et al.* 1993), on the demonstration of receptors for GH (Hirano, 1991; Gray *et al.* 1990; Sakamoto and Hirano, 1991) and on the regulation of GH-producing cells (Marchant *et al.* 1987; Nishioka *et al.* 1988). A pivotal target for GH is the liver (Hirano, 1991), where it stimulates receptors with nanomolar affinity. Liver cells activated by GH produce a fish insulin-like growth factor (IGF, probably IGF-I; Drakenberg *et al.* 1989) that may subsequently stimulate growth (Duan and Hirano, 1990; Sakamoto and Hirano, 1991; Gray and Kelley, 1991; McCormick *et al.* 1992*a*,*b*). IGF also has osmoregulatory actions, as has recently been detected in rainbow trout by McCormick *et al.* (1991). The demonstration of GH receptors in rainbow trout gills (Sakamoto and Hirano, 1991) and of an osmoregulatory role for IGF suggests that an analogous 'GH–IGF system' is present in gills and liver. The apparent low density of GH receptors in branchial epithelium ($3.1\,fmol\,mg^{-1}$ protein) should be interpreted with care: assuming that the ionocytes in the gills are the target for GH and an average ionocyte density of 3% in the branchial epithelium (G. Flik, personal observation on freshwater rainbow trout and tilapia), the receptor density of ionocytes ($3.1\times100/3=103\,fmol\,mg^{-1}$ protein) may be even higher than that of hepatocytes ($71.0\,fmol\,mg^{-1}$ protein). Clearly, the presence of GH receptors on ionocytes is in good agreement with the consensus that GH exerts osmoregulatory effects in seawater-adapting and seawater-adapted salmonids, and this action could be independent of IGF production by the liver.

To our knowledge, only one study has been published on the effects of GH in tilapia (Clarke *et al.* 1977): in juvenile tilapia, high doses of homologous GH stimulated growth, as was indicated by increases in length and mass. We have shown before (Flik *et al.* 1985, 1986*a*,*b*) that, in tilapia, calcium uptake from the water *via* the gills is pivotal for growth and calcium homeostasis. The availability of recombinant tilapia GH (rtGH; Rentier-Delrue *et al.* 1989) allowed us to test homologous GH for its growth-promoting effect in freshwater tilapia. We focused our study on the effects of rtGH on calcium handling by the tilapia. Freshwater tilapia were weighed and injected with rtGH twice a week for up to 7 weeks. We measured the hepatosomatic index and the calcium balance of the fish and analyzed the plasma ion composition, the opercular ionocyte density and the bone and scale calcium and phosphate contents.

Materials and methods

Fish

Tilapia, *Oreochromis mossambicus* Peters, were obtained from laboratory stock. The fish were held in 120-l all-glass aquaria with a continuous flow of Nijmegen city tapwater ($0.7\,mmol\,l^{-1}$ Ca^{2+}, $0.2\,mmol\,l^{-1}$ Mg^{2+}, $0.5\,mmol\,l^{-1}$ Na^{+}, $0.06\,mmol\,l^{-1}$ K^{+}, pH7.8);

the water temperature was 26±0.2°C. Lights were on for 12h per day. The loading density of fish did not exceed 1.5kg100 l^{-1}. Commercial trout pellets (Trouvit, Putten, The Netherlands) were administered by means of automated food dispensers throughout the light period at 2 or 5% of the recorded total mass of the groups of fish per day. Care was taken that all fish had access to the food and that all food was consumed. Experiments were carried out in May and June.

Hormone treatment and experimental design

Recombinant tilapia growth hormone (rtGH; Rentier-Delrue *et al.* 1989) was dissolved in 0.02mol l^{-1} NH_4HCO_3 (pH9.0) and diluted to the required concentration with phosphate-buffered saline (pH7.8). The dose was 100ng g^{-1} fish. Injections were given intraperitoneally with a Hamilton precision syringe. The carrier volume was 1 μl g^{-1} fish. Control animals received saline injections. Fish were injected twice a week, on Monday morning and Thursday afternoon. The fish were weighed before injection. The use of a Mettler PM34 Delta range balance allowed weighing of free-swimming fish and stress is therefore minimized compared with that seen with netting. Four groups of 15 fish each were included in an experiment: one group was weighed at the start and upon completion of the experiment, and not handled further (group U); one group was handled in the same way as the injected groups, but not injected (group H); one group served as injection controls (group S); and one group received rtGH injections (group GH). The holding conditions for all four groups were identical. The duration of the hormone treatment was 44 days.

Upon completion of the experiments, the fish were quickly anaesthetized in Tris-buffered (pH7.8) 3-aminobenzoic acid ethyl ester (MS-222; 1 g l^{-1}) and blood was collected (see below); subsequently, the fish were killed by spinal transection. The liver was removed and weighed to calculate the hepatosomatic index, defined as $(W_l/W_f)\times 100\%$ (where W_l is the mass of the liver in grams and W_f is the mass of the fish in grams). One operculum was removed for determination of the ionocyte density. Vertebral bone and scale samples were collected to assess calcium and phosphate contents (see below).

Plasma analysis

Mixed arterial and venous blood was collected by puncture of the caudal vessels, using a heparinized tuberculin syringe fitted with a 23-gauge needle. Plasma was separated from cells by centrifugation (1min, 9000 ***g***). Part of the plasma was ultrafiltered (Millipore Ultrafree-MC, molecular cut-off 10000Da) for the estimation of free calcium and magnesium levels. Total calcium and magnesium of plasma and ultrafiltered plasma were determined with commercial colorimetric kits (Sigma, St Louis, MO, USA). Combined calcium/phosphate and magnesium standards (Sigma) were used as reference. Plasma sodium and potassium contents were determined by flame emission spectrophotometry. Plasma osmolality was determined with a Roebling osmometer on fresh 50 μl plasma samples. Cortisol was determined on 5 μl of plasma by radioimmunoassay (Amerlex assay IM-2021, Amersham plc, UK), which is based on a highly specific antibody. The assay is not affected by the anticoagulant heparin. The

recovery, defined as the increase in value when a known amount of cortisol is added to the sample and expressed as the percentage recovery (i.e. the measured increase divided by the predicted increase), varied between 98% and 103% in the range of the standard curve. The measurement range of this assay is 0–1700nmol l^{-1}. The sensitivity of the standard curve, estimated from the 95% confidence intervals of the within-assay variation of the zero standard counts, was 3±1nmol l^{-1} (*N*=5). Serial dilutions of plasma samples yielded curves parallel to the standard curve.

Bone calcium and phosphate

Triplicate samples of 10 scales each were taken from both sides at the mid-lateral region, posterior to the operculum. A sample of vertebral bone was taken after removal of adhering muscle tissue by microwave cooking (1min, 700W). All tissues were weighed and dried to constant mass at 90°C, and the dry mass was determined to the nearest 0.01mg. Dried samples were dissolved in 0.5ml of concentrated HNO_3 at 60°C for 1h. The sample volume was brought to 5ml with double-distilled water. The total calcium was determined by a Thymol Blue method (for details, see Flik *et al.* 1986*a*) and phosphate was determined according to Fiske and Subbarow (1926).

Opercular ionocyte density

Opercula were incubated for 1h in 2 μmol l^{-1} 2-(dimethylaminostyryl)-1-ethylpyridinium iodine (DASPEI) dissolved in water. DASPEI stains the mitochondrion-rich chloride cells (ionocytes) (Bereiter-Hahn, 1976). After rinsing, the inner opercular epithelium was examined in a Zeiss fluorescence microscope at a magnification of 250×. Cells were counted in 20 different squares of the opercular epithelium with a total surface area of 5mm^2 per fish.

Calcium fluxes

The rtGh-treated fish and their controls were used 44–46 days after the start of hormone treatment. Unidirectional calcium influx was determined on the basis of initial uptake rates of $^{45}Ca^{2+}$ from the water and the water $^{45}Ca^{2+}$ specific activity, as described in detail elsewhere (Flik *et al.* 1989). Briefly, fish were exposed to water containing $^{45}Ca^{2+}$ of known specific activity. The fish were not fed during the flux determination. After 4h, the fish was anaesthetized, rinsed in 10mmol l^{-1} $CaCl_2$ to remove tracer adsorbed to the body surface and quick-frozen on solid CO_2. Next, the fish was partly defrosted and the still frozen intestinal tract was removed. Subsequently, the $^{45}Ca^{2+}$ content of the fish and intestinal tract was determined after microwave-cooking and blending the cooked tissues with a known amount of distilled water in a Waring-type blender. Using this method, tracer uptake as a result of drinking is separated from branchial tracer uptake (Pang *et al.* 1980). The whole-body calcium influx determined in this way was shown to reflect extraintestinal branchial influx. Growing tilapia increase their total body calcium pool, and its size is directly related to the mass of the fish (see below). Therefore, net calcium influx may be determined on the basis of the growth-related increase in the total body calcium pool (the net influx equals the mean accumulation rate of calcium). The treatments reported here did not affect the calcium content of the bone, and therefore the

total body calcium pool (Q_f) may be calculated as $Q_f = 357.5 W_f^{0.965}$ μmol (W in g; Flik *et al.* 1985). Net calcium influx was calculated as $\Delta Q_f/\Delta t$ (time in h) over the 44 day period of the experiment. Calcium efflux (F_{out}) was calculated as the difference between branchial calcium influx (F_{in}) and net calcium influx (F_{net}), $F_{out}=F_{in}-F_{net}$.

The fish used in the experiments received food at a dose of 2 or 5% of their mass per day. To evaluate the effects of the amount of food provided on the calcium influx from the water, a comparison was made between the measured calcium influx and the predicted calcium influx at a food dose of 1% per day, given by the equation: $F_{in}=50 W_f^{0.805}$ nmol h^{-1} (W in g; Flik *et al.* 1985).

Statistics

Values are presented as means ± S.D. Differences among groups were assessed by means of a one-way analysis of variance. Significance of differences between means was subsequently assessed by the Newman–Keuls test or the Mann–Whitney *U*-test, where appropriate. Linear regression analysis was based on the least-squares method. Significance was accepted when $P<0.05$.

Results

rtGH treatment significantly stimulated growth as determined by the increase in body mass and length after 44 days (Table 1). These increases were significant when compared with those of unhandled ($P<0.01$) as well as with those of handled and saline-injected groups ($P<0.05$). No differences were observed among the control groups (U, H and S). The percentage increase in body mass over the initial mass after 44 days of treatment was 44.9% for the GH group and 25.6, 28.1 and 28.2% for groups U, H and S, respectively. The percentage increase in length was 15% for GH-treated fish and 8.4%, 9.1% and 9.1% for groups U, H and S, respectively.

Branchial calcium influx increased with the amount of food provided (Table 2). Male

Table 1. *Effects of recombinant tilapia growth hormone on body mass (in g) and length (in cm) of freshwater tilapia*

	Day				
	0		20	44	
Group	Mass	Length	Mass	Mass	Length
U	65.3±14.2	15.2±1.1	76.5±18.8	82.0±13.0	16.5±1.0
H	64.5±12.1	15.2±0.9	78.7±15.4	82.6±9.1	16.5±0.7
S	64.1±12.2	15.1±0.9	75.2±15.4	82.2±14.3	16.5±1.1
GH	67.9±11.7	15.4±0.9	83.2±14.6	98.4±6.2*	17.7±0.5*

U, unhandled; H, handled; S, saline-injected; GH, rtGH-injected fish.
Significant differences were observed after 44 days of treatment.
*Significantly different from H ($P<0.01$) and U and S ($P<0.05$; Newman–Keuls test).
Mean values ± S.D. are given for 9–15 fish per group.

Table 2. *Feeding and branchial calcium influx in freshwater tilapia*

Food	Sex	*N*	W_f (g)	F_{in} (measured)	F_{in} (calculated)
2%	Male	12	108±9	5.80±2.64*	2.01±0.71
5%	Male	14	87±21	11.71±7.14†	2.09±0.76
5%	Female	7	64±20	6.20±3.82	2.22±0.18

F_{in} is expressed in $\mu mol\,h^{-1}\,100\,g^{-1}$ fish; F_{in} (calculated) according to $F_{in}=0.05M_f^{0.805}$ ($\mu mol\,h^{-1}$), W_f in g, applying to tilapia fed 1% food.

Values are mean ± S.D.

*Significantly different from males fed 5% food ($P<0.01$, Mann–Whitney *U*-test); †significantly different from female fish fed 5% food ($P<0.05$, Mann–Whitney *U*-test).

fish fed 5% per day had a calcium influx twice as high ($P<0.05$, Mann–Whitney *U*-test) as male fish fed 2%, 11.7 and 5.8 $\mu mol\,h^{-1}\,100\,g^{-1}$, respectively. Calculated values for branchial calcium influx at a food dose of 1% are around 2 $\mu mol\,h^{-1}\,100\,g^{-1}$. For male fish, the uptake of calcium from the water was positively correlated with the amount of food consumed according to: $F_{in}=(2.36\pm0.18)\times(\%\ food)+0.11\ \mu mol\,h^{-1}\,100\,g^{-1}$ ($r=0.987$; $P<0.01$). Female fish had a calcium influx significantly ($P<0.01$, Mann–Whitney *U*-test) lower than that of male fish [both fed 5%, 6.2 and 11.7 $\mu mol\,h^{-1}\,100\,g^{-1}$, respectively]. The 5% food regimen represented *ad libitum* food conditions and was chosen for the GH experiment.

rtGH had no effects on calcium influx from the water (Table 3). As a result of the increased growth rate, rtGH-treated fish showed an enhanced net accumulation of calcium (9.45±0.60 $\mu mol\,h^{-1}\,100\,g^{-1}$), 36% higher than in the untreated fish ($P<0.025$) and 25% higher than in the handled fish ($P<0.05$) and 37% higher than in the saline-injected fish ($P<0.01$). The calculated net efflux of calcium in rtGH-treated fish was, therefore, lower than in the controls.

Table 3. *Growth hormone and calcium fluxes in freshwater tilapia*

Group	F_{in}	F_{net}	F_{out}
U	10.51±3.0	6.93±1.10	3.58
H	10.21±2.8	7.55±0.83	2.66
S	10.80±4.1	6.88±1.20	3.92
GH	11.31±3.0	9.45±0.60*	1.86

Fluxes are given in $\mu mol\,h^{-1}\,100\,g^{-1}$ fish; values are mean ± S.D.

F_{in} was determined on the basis of $^{45}Ca^{2+}$ influx from the water; F_{net} was calculated on the basis of the increase in mass and the total body calcium pool of the fish in time and the relationship between the total body calcium pool and fish mass, $Q_f=357.5W^{0.965}$ μmol (*W* in g).

$F_{out}=F_{in}-F_{net}$.

GH treatment was for 44 days (for details see Materials and methods section).

Fish were fed 5% food per day.

*Significantly different from groups U ($P<0.025$) and S ($P<0.05$) and H ($P<0.01$; Newman–Keuls test).

Table 4. *Effects of growth hormone on plasma ion composition and cortisol levels*

	Controls	GH
Total calcium ($mmol\,l^{-1}$)	3.09±0.39	3.00±0.55
Ca^{2+} ($mmol\,l^{-1}$)	1.26±0.14	1.28±0.33
Total magnesium ($mmol\,l^{-1}$)	1.10±0.20	1.09±0.08
Mg^{2+} ($mmol\,l^{-1}$)	0.55±0.08	0.51±0.12
Na^{+} ($mmol\,l^{-1}$)	130±12	132±16
Osmolality ($mosmol\,kg^{-1}$)	302±8	298±16
Cortisol ($nmol\,l^{-1}$)	1050±448	539±224*

Mean values ± S.D. are given for 20 (controls) and 15 (GH) fish.
Fish were treated for 44 days with rtGH (for details see Materials and methods section).
*Significantly different from controls (P<0.025; Mann–Whitney U-test).

Table 4 gives data on plasma ion composition and cortisol levels in fish treated for 44 days with rtGH. The data for the three control groups (U, H and S) have been pooled as no differences were observed among these groups for any of the variables measured. GH did not influence plasma ion composition but had a significant (P<0.025, Mann–Whitney U-test) effect on plasma cortisol levels, which were almost 50% lower than in the controls.

GH treatment increased the hepatosomatic index (HSI) compared with that of saline-injected (P<0.01) and handled and untreated fish (P<0.05) (Fig. 1). No differences were observed among groups U, H and S. In GH-treated fish, the HSI increased by 43 % compared with that of saline-treated fish (from 1.64 to 2.34%).

Analysis of vertebral bone and scales (Table 5) revealed no differences among the four groups of fish (U, H, S and GH) after 44 days of treatment. The values for the calcium

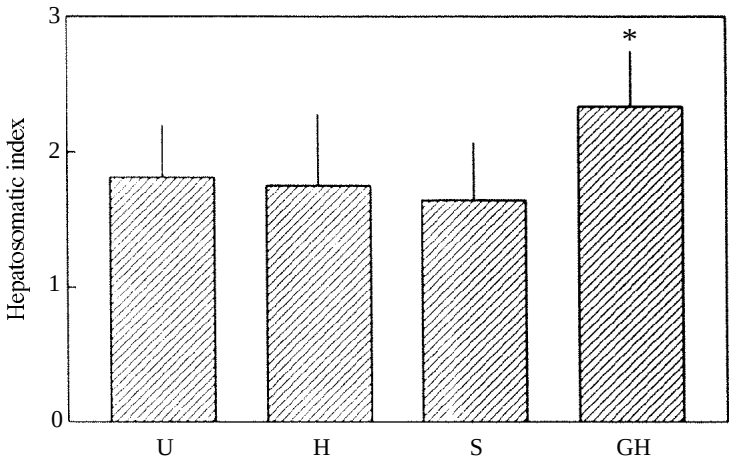

Fig. 1. Effects of GH on the hepatosomatic index [(mass of liver/mass of fish)×100%] after 44 days of treatment with GH. U, unhandled fish; H, handled fish; S, saline-injected fish; GH, growth-hormone-injected fish. *Significantly different from S (P<0.01) and U and H (P<0.05; Newman–Keuls test. Bars indicate standard deviation; N=9.

Table 5. *Recombinant tilapia growth hormone and bone calcium and phosphate content*

Group	Calcium (mmol g^{-1})	Phosphate (mmol g^{-1})	Ratio
Scales			
U	5.38±0.66	3.74±0.50	1.44±0.08
H	5.03±0.38	3.73±0.31	1.35±0.08
S	5.03±0.50	3.62±0.34	1.39±0.13
GH	4.80±0.62	3.74±0.19	1.28±0.17
Vertebrae			
U	5.25±0.36	3.62±0.21	1.45±0.07
H	5.16±0.50	3.71±0.3	1.39±0.06
S	5.20±0.48	3.98±0.34	1.31±0.03
GH	5.04±0.36	3.68±0.37	1.37±0.09

Mean values ± S.D. are given for nine fish per group.
Fish were treated with rtGH for 44 days as described in detail in the Materials and methods section.

content of vertebrae and scales are similar to previously reported values for tilapia (Flik *et al.* 1986*b*). The ratios of calcium to phosphate are in all cases close to the value of 1.43, representative for whitlockite, the probable form of apatite in fish bone (Herrmann-Erlee and Flik, 1989).

Opercular ionocyte densities increased in GH-treated fish compared with all other groups ($P<0.01$) (Fig. 2). The ionocyte density in the saline-injected group was significantly higher than in the unhandled ($P<0.01$) and handled ($P<0.05$) groups.

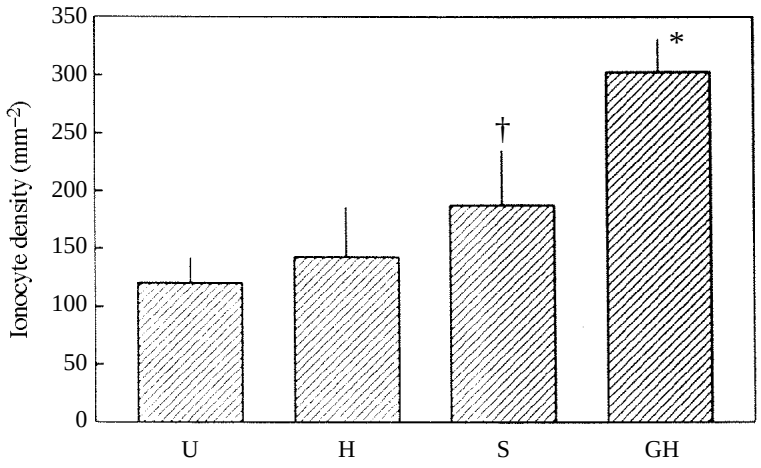

Fig. 2. Effects of GH on opercular ionocyte density after 44 days of GH treatment. U, unhandled fish; H, handled fish; S, saline-injected fish; GH, growth-hormone-injected fish. *Significantly different from U, H and S ($P<0.01$); †significantly different from U ($P<0.01$) and H ($P<0.05$; Newman–Keuls test). Bars indicate standard deviation; $N=9$.

Discussion

The results presented here demonstrate that tilapia recombinant growth hormone (Rentier-Delrue *et al.* 1989) is bioactive. The mild growth-promoting activity of rtGH in freshwater tilapia compares well with the mild effects of homologous recombinant GH in carp (Fine *et al.* 1993). The growth-stimulatory activity is further substantiated by its classical stimulatory effect on the hepatosomatic index. The growth-promoting effect of rtGH, therefore, appears to be mediated through stimulation of the liver, where the IGF is produced that subsequently enhances the growth of cartilage and bone. No change in the plasma ion composition was observed after rtGH treatment. The bone compartments increased in size but no changes in the mineral density occurred. Apparently, sufficient calcium, phosphate and bone matrix were available for calcium deposition to allow enhanced growth without influencing plasma ion composition or bone mineral density. The effect of GH on the calcium balance of tilapia is clearly different from that of prolactin (PRL). PRL does not stimulate growth, but stimulates calcium influx and reduces calcium efflux and, by doing so, induces hypercalcaemia and increases bone calcium density (Flik *et al.* 1986*b*; Swennen *et al.* 1991). We have reported before that low water levels of calcium (i.e. 0.2mmol l^{-1} or less) may influence bone calcium content and plasma ion composition in tilapia (Flik *et al.* 1986*a*; Urasa and Wendelaar Bonga, 1987). Furthermore, it has been shown that tilapia mobilize calcium and phosphate from their bone, but only in times of shortage, e.g. during ovarian maturation and when diets are deficient in calcium (Urasa *et al.* 1985). It appears that fish do not show the strict calcium homeostasis observed in terrestrial vertebrates and rely primarily on external calcium (Perry and Flik, 1988). The observation that the unidirectional influx of calcium from the water is always larger than the calculated net influx (i.e. the growth-related calcium accumulation rate) into the fish suggests that fish rely on branchial calcium influx for growth and homeostasis: the amount of calcium offered in the food would have been sufficient to allow the growth observed (see below), yet the fish increased their branchial calcium influx. We realize that an estimation of the efficiency of intestinal calcium absorption may ultimately shed light on the role of intestinal calcium handling for growth.

A remarkable observation is that branchial calcium influx increased linearly with the amount of food offered to the fish. Given a food calcium content of 850 μmol g^{-1} at an amount of 5% of its body mass per day, a 100g fish obtains $5\times850=4250$ μmol calcium. The Ca^{2+} influx *via* the gills per day is $F_{in}\times24=11.7\times24=280.8$ μmol for a 100g fish. Thus, although the food-associated calcium would suffice to cover the fish's extra need for (enhanced) growth, a larger dietary calcium input does not lead to a decreased branchial calcium influx. Ample evidence has been given that calcium transport occurs in the proximal intestine of the tilapia (Flik *et al.* 1990). However, we have no information on the net movements of calcium in more distal segments of the intestine, where calcium secretion must occur. The role of dietary calcium in the calcium metabolism of the fish remains enigmatic: a calcium-deficient diet does not hamper calcium homeostasis or growth (Berg, 1970; Urasa and Wendelaar Bonga, 1987). Another line of evidence suggesting that branchial calcium uptake is adjusted to the growth rate of the fish comes

from our data on female fish: under identical conditions, female fish have a lower calcium influx from the water, grow more slowly and do not reach the size of males (in our laboratory).

Recombinant tilapia GH did not enhance calcium influx (F_{in}) from the water. We realize that this statement is based on data that reflect a single time point, i.e. 44 days after the start of the treatment. It could well be that the unidirectional influx was stimulated earlier in the 44-day period. Such an increase could have accounted for an increased integrated net flux over the 44-day period, whereas the increased unidirectional influx had attenuated by day 44. The conclusion would be that rtGH transiently stimulated Ca^{2+} influx from the water to enhance calcium accumulation for growth. However, on the basis of the consistent stimulation of growth observed in this experiment, we consider this possibility to be unlikely. Moreover the Ca^{2+} influxes determined 16 days after the start of treatment on six saline-injected and five rtGH-injected fish were not significantly different (F_{in}=8.9±3.4 and 10.3±4.1 $\mu mol\,h^{-1}\,100\,g^{-1}$ fish, for saline- and GH-treated fish, respectively; P>0.10), nor were these values different from those determined after 44 days of treatment. The Ca^{2+} influx in a fish provided with 5% food per day appears to be high enough to cover the growth-related accumulation of calcium. The rtGH-treated fish grew faster and enlarged their bone compartment (the bone calcium content did not change); these fish apparently accumulated more calcium per unit time (F_{net} increased). As a result, the calculated total efflux of calcium is lower in rtGH-treated fish. Direct measurements of branchial and extrabranchial calcium efflux are required to discriminate between branchial, renal and intestinal efflux routes as targets for rtGH.

The stimulation by rtGH of the opercular, and presumably the branchial, ionocyte density suggests an osmoregulatory action of the hormone. The total, DASPEI-stainable ionocyte population is not necessarily a good indicator of ion-transport activity of the gills. It may well be that the ionocyte turnover and number were stimulated by rtGH, but that at the same time the number of functional ionocytes, i.e. fully developed cells in contact with the water (Wendelaar Bonga *et al.* 1990), remained unchanged. However, we favour the idea that this rtGH effect in freshwater fish was an IGF-dependent mitogenic effect of exogenous GH. Clearly, these data require future evaluation of IGF levels. A stimulation of branchial ionocytes has been reported for seawater trout (Madsen, 1990); injection of GH into seawater fish improves hypo-osmoregulatory mechanisms and therefore the stimulation by GH of branchial ionocytes may be of functional significance.

The opercular ionocyte density in the control groups was somewhat higher than the densities reported before, 120–188mm^{-2} *versus* less than 100mm^{-2} in control freshwater tilapia (Wendelaar Bonga *et al.* 1990). We tentatively relate this increased ionocyte density to the relatively high cortisol levels (discussed below) seen in these fish (around 1000$nmol\,l^{-1}$). However, an increased cortisol level does not explain the increased ionocyte density in the GH-treated fish, as cortisol levels were about 50% lower in these fish than in the controls. It would be interesting to know the dynamics of cortisol under these conditions. The lower level of cortisol could reflect an increased utilization of cortisol in rtGH-treated fish.

In general, the plasma cortisol levels in all four groups were high compared with those

of completely unstressed fish (typical cortisol levels around 100nmoll^{-1}; G. Flik, unpublished observation). The present experimental set-up with frequent handling and an intensive feeding regimen, in all likelihood, leads to increased cortisol levels. Indeed the levels in groups U, H and S are elevated to a level normally seen in fish 10min after the start of netting. An alternative explanation for the lower cortisol levels in the GH group may be that the stress-related cortisol response in these fish has become suppressed. We did not observe differences between unhandled and handled control fish with respect to cortisol levels and, therefore, tentatively conclude that the elevated plasma cortisol levels somehow relate to the high dose of food provided for our fish. Again, this topic needs further research.

Lower cortisol levels in GH-treated fish may further indicate that GH exerts a negative control over the interrenals, a conclusion in line with the data of Carsia *et al.* (1985) on domestic fowl. It contrasts, however, with data on coho salmon by Young (1988), who reported a stimulatory action of (ovine) GH on the interrenals, both *in vivo* and *in vitro.* A negative feedback of GH on the interrenals, as suggested by our data, completes the functional interrelationship between cortisol and GH-producing cells proposed by Nishioka *et al.* (1985), who reported stimulatory effects of cortisol on GH release by tilapia pituitary gland *in vitro.*

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